Where's the *Bt*?

Tracking the Protein's Fate During Ethanol Production

As ethanol's production as a cleaner-burning alternative to petroleum fuels gains momentum, the research of Bruce S. Dien's team in Peoria, Illinois, couldn't have come at a better time.

In fermentation experiments at ARS' National Center for Agricultural Utilization Research, Dien's team charted the biochemical fate of *Bt* protein during both wet- and dry-milling of genetically modified (GM) corn into ethanol and other co-products. His team also compiled early evidence that corn's ethanol yield is influenced by how easily its starch breaks down as well as by how much starch it contains.

"In our research with traditional alcohol fermentations, we found that the digestibility of starch influenced ethanol production efficiency as much as total starch content did, and that *Bt* protein in GM corn was not detectable after fermentation," says Dien, a chemical engineer in the Fermentation Biotechnology Research Unit (FBRU).

Both topics, he adds, are of interest to seed companies and corn farmers alike as they strive to tap into new, specialty-use markets for the grain crop. Ethanol is deemed a promising product as increasing concern over U.S. dependency on foreign oil has intensified the search for viable alternatives. (See "Bioenergy Today," *Agricultural Research*, April 2002, p. 4.)

Are ethanol yields from *Bt* corns the same as those from non-*Bt* corns?

Bt+Starch=?

Bt stands for *Bacillus thuringiensis*, a soil bacterium whose genes for making the protein have been copied and spliced into corn plant DNA as a natural, built-in insecticide. Among the chief targets of this biotechnological defense is the European corn borer, a moth whose caterpillar stage costs U.S. farmers an estimated $1 billion annually in losses.

Bt corn has gone from less than one-half million acres planted in 1996—the year of its commercial debut—to 19.5 million acres in 2000, the latest year for which the Environmental Protection Agency has statistics. Yet, despite the good attributed to *Bt*, such as cost savings and environmental benefits from reduced use of chemical insecticides, there's been little research on the protein's fate in corn during processing into ethanol.

And with ethanol production forecasted to increase from the current 1.7 billion gallons to 4 billion gallons by 2006, the effect of *Bt* corn on the biofuel's production presents something of an X-factor, or unknown variable.

Are *Bt* ethanol yields the same as those from non-*Bt* crops? And if *Bt* protein shows up during ethanol production, by what methods could refiners detect and quantify it? These are some of the questions posed to scientists by industry.

SCOTT BAUER (K9909-1)

Microbiologist Nancy Nichols and biochemical engineer Bruce Dien add yeast to a bioreactor to begin ethanol fermentation. *Bt* and non-*Bt* corn hybrids were compared for ethanol yields.

"There isn't a lot of research information out there because the widespread use of *Bt* in corn is still relatively new," says Rodney J. Bothast, a microbiologist at the FBRU. "And since some *Bt* corn does enter ethanol production plants, we wanted to see what happens to it."

So, in 2000, Dien, Bothast, and ARS microbiologist Loren B. Iten teamed up with two other researchers, Lynda Barrios and Steven R. Eckhoff, who had already done some preliminary wet- and-dry milling studies at the University of Illinois Department of Agricultural Engineering in Urbana-Champaign.

Together, they designed small-scale, wet- and dry-milling experiments that would allow them to monitor *Bt* protein of two modified corn hybrids during all stages of ethanol production. They also compared the *Bt* corns' overall ethanol yield to that of non-*Bt* versions of the two hybrids. During each stage of the experiment, says Bothast, "we basically repeat in the lab what happens in an actual ethanol plant."

A hybrid containing lots of easily digested starch can translate into greater returns on a crop for the ethanol market.

Steeped in *Bt*?

Wet-milling involves several steps, starting with soaking, or steeping, corn in water and sulfur dioxide for 24 to 36 hours. During later stages, the corn is ground up to facilitate separation of its starch, fiber, germ oil, and protein. Glucose and other simple sugars derived from the starch fraction are fermented inside giant vats containing live cultures of *Saccharomyces cerevisiae* yeast.

"Starch is made up of glucose molecules hooked to one another by a chemical bond known as an alpha-1,4 linkage," explains Bothast. "During fermentation, the yeast takes up this glucose molecule, metabolizes it, and converts it into two products: carbon dioxide and alcohol."

In dry-milling, ground cornmeal is cooked during a liquefaction stage with water and an enzyme, alpha-amylase. The enzyme helps breaks down the starch fraction into smaller parts and keeps it from becoming a gel. What's left is a mash that's later fermented into ethanol.

For their studies, Dien's team used 300-milliliter flasks or 2.5-liter or 30-liter bioreactor tanks to ferment corn from two *Bt*-modified hybrids and two non-*Bt* ones.

At each stage they checked for a *Bt* protein—a type known as CRY1Ab—using an antibody-based test known as ELISA, short for enzyme-linked immunosorbent assay.

Dien says their test results indicate that use of heat during dry-milling tends to denature, or destroy, *Bt* protein.

Tests of cornmeal from the two *Bt* hybrids revealed CRY1Ab protein concentrations of 196 parts per billion (ppb). But once that meal is liquefied, "the *Bt* disappears in less than 15 minutes," the scientists report. And there was no detectable trace of it in either the mash or the resulting ethanol.

A slightly different story unfolded for the wet-milled corn. While samples of whole kernels, gluten, germ oil, and fiber contained *Bt* protein at concentrations of 170 to 453 ppb, no trace could be found in the starch or steep liquor fractions. Nor should it appear later on in the ethanol, they say. That's because the *Bt* protein gets separated from the starch with the other proteins. (The researchers note that use of high temperatures to dry corn gluten meal and corn gluten feed may eliminate any protein that survives the wet-milling process.)

Bt and non-*Bt* corn hybrids yielded about the same amount of ethanol, and the yields were comparable to those achieved in industrial production. On average, a bushel of corn (56 pounds wet weight) yields about 2.7 gallons of ethanol via dry-milling versus 2.5 for wet-milled corn, notes Dien. The team will publish the findings in an upcoming issue of the journal *Cereal Chemistry.*

Heavy on Starch

In that same paper, they'll also report preliminary results of a follow-up study that challenges the common assumption that corn hybrids with high starch contents yield the most ethanol.

"The take-home message here is that not all starches are created equal," says Bothast.

In the study, scientists fermented corn samples from five hybrids containing between 68 and 72 percent starch. Using a standard method, they measured the hybrids' fermentation efficiency (CE). This refers to how much of the total starch actually breaks down into the glucose sugar that gives rise to ethanol.

Representative of the study's findings are the ethanol yields and CE rating for hybrid A and hybrid C. Hybrid A, with a starch content of 68 percent, yielded 2.73 gallons of ethanol per bushel, with a CE rating of 92 percent. Hybrid C contained 72 percent starch, and yielded 2.83 gallons of ethanol per bushel. But its lower CE rating, 90 percent, meant that less of that starch actually converted into sugar, the researchers say.

Bothast considers the findings preliminary since the five hybrids they tested are a small sampling of the hundreds now grown commercially. They also want to further verify the methods by which they obtained their results, as the research eventually could provide commercial seed companies with a protocol they could use in their corn breeding programs.

SCOTT BAUER (K9910-1)

Microbiologist Loren Iten operates a recirculating batch jet cooker used for corn-to-ethanol research studies. The batch jet cooker models the liquefaction step in the dry-milling ethanol process.

Farmers, too, are keeping close tabs on the scientists' work, since a hybrid packing lots of easily digested starch can translate into greater returns on their crop for the ethanol market. Likewise, "the seed companies will want to be able to show, this is our best hybrid for making ethanol," says Bothast.

But what actually makes a hybrid's starch readily break down into simple sugars for easier fermentation remains a mystery.

"That's the million-dollar question," says Bothast. "It could be due to the environment in which the corn is grown, or the DNA comprising its genes and their subsequent effect on the composition of the corn itself."

Yeast Power

Perhaps the unsung heroes in this push for peak ethanol production are the *Saccharomyces* yeast organisms.

"They've been the workhorses of the industry and are absolutely our best friends," the microbiologist says. In fact, a chief emphasis of the Peoria team all along has been maximizing the yeast's ability to efficiently convert carbohydrates to new, value-added co-products.

"Our goal is to have a fermentation organism that uses all kinds of sugars—not just those from starch, but also from the fiber," says Bothast. "Theoretically, you could get a 10-percent increase in ethanol production" from using these other sugars.—By **Jan Suszkiw,** ARS.

This research is part of Quality and Utilization of Agricultural Products, an ARS National Program (#306) described on the World Wide Web at http://www.nps.ars.usda.gov.

Bruce S. Dien, Rodney J. Bothast, and Loren B. Iten are in the USDA-ARS Fermentation Biotechnology Research Unit, National Center for Agricultural Utilization Research, 1815 N. University St., Peoria, IL 61604; phone (309) 681-6270 [Dien], -6566 [Bothast], -6210 [Iten], fax (309) 681-6427, e-mail dienb@mail.ncaur.usda.gov, fbrjb@mail.ncaur.usda.gov, itenlb@mail.ncaur.usda.gov. ◆